

# Lysozyme activity in ultrastructurally defined fractions of alveolar macrophages after inhalation exposure to nickel

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**ABSTRACT** Rabbits were exposed to 0.6 mg/m<sup>3</sup> of nickel as NiCl<sub>2</sub> for about one month. After exposure, alveolar macrophages were lavaged from the lung and divided into three fractions by elutriation. Laminated structures in the macrophages were related to fraction number so that the fractions with the largest cells contained the highest number of structures. The lysozyme activity decreased in unfractionated as well as in fractionated macrophages from nickel exposed rabbits. The decrease was most pronounced in the fraction with the smallest macrophages and smallest number of laminated structures. Therefore the pronounced decrease in lysozyme activity seen in this and earlier studies is not caused by the increased amount of surfactant material. Increased amount of surfactant is a hallmark of nickel inhalation exposure and the surfactant material is responsible for the morphological and metabolic effects of the macrophages. The decreased lysozyme activity is probably a direct effect of nickel on the macrophages.

Inhalation exposure of rabbits to low levels (0.1–1 mg/m<sup>3</sup>) of nickel in metallic or soluble form from one to eight months affects alveolar epithelial type II cells; increases the content of phospholipids in the lung, especially disaturated phosphatidylcholines; and alters the morphology and oxidative metabolic activity of alveolar macrophages.<sup>1</sup> These effects are produced by nickel concentrations similar to, and sometimes even below, the present occupational threshold limit values (TLVs). In the United States the TLV for metallic nickel is 1 mg/m<sup>3</sup> and for soluble nickel 0.1 mg/m<sup>3</sup>. The pattern of effects is similar to that seen in rats after exposure to high concentrations of quartz dust and also similar to the pathological picture seen in the human disease, pulmonary alveolar proteinosis.<sup>2-4</sup> Neither metallic iron, cobalt or chromium dust, nor chlorides of copper, cobalt, or manganese produce the same effect pattern.<sup>1,5</sup> Cadmium chloride, however, does produce a similar effect of alveolar type II cells and lung content of lipids as nickel chloride; in addition, Cd<sup>2+</sup> causes interstitial inflammation.<sup>6</sup>

Exposure of rabbits to low levels (0.1 mg/m<sup>3</sup>) of

metallic nickel or nickel chloride aerosols greatly decreases the lysozyme activity in lung lavage fluid and in macrophages<sup>7,8</sup> whereas lysozyme activity increases or is unchanged after exposure to chlorides of cadmium, copper, or cobalt. Lysozyme is bacteriolytic to most Gram positive bacteria and acts synergistically with other immunological mechanisms with other bacteria.<sup>9-12</sup>

Exposure to nickel produces a specific effect pattern. This effect pattern is restricted to changes in alveolar epithelial type II cells and alveolar macrophages and an increase in surfactant material. In vitro experiments indicate that several of the effects on the macrophages are caused by the increased amount of surfactant<sup>13</sup>—for example, certain morphological changes and an increase in metabolic activity, as determined by measuring the macrophages ability to reduce nitroblue tetrazolium (NBT) to formazan. The aim of the present study was to investigate the lysozyme activity in macrophage fractions containing different amounts of laminated inclusions to see whether or not changes in lysozyme activity were related to the increase in surfactant material in the cells. Exposure to nickel produces large variations in the cell size of alveolar macrophages and there is an

increase in laminated structures, especially in the larger macrophages.<sup>14,15</sup> The nitroblue tetrazolium activity was also studied in the macrophage fractions as a positive control.

## Material and methods

### EXPOSURE DATA

Sixteen male rabbits, weighing  $2.6 \pm 0.3$  kg, were divided into two groups of eight animals each. One group was exposed to  $0.6 \pm 0.2$  mg/m<sup>3</sup> of Ni<sup>2+</sup> as NiCl<sub>2</sub> and the other group constituted a control group and was exposed to filtered air only. During the exposure period, which was four to six weeks, five days a week, six hours a day, exposed animals and controls were kept in exposure chambers, 0.6 m<sup>3</sup> in size.<sup>16</sup> The concentration of nickel was measured by air suction through a membrane filter (Gelman Gn-4, 0.8 µm) and the amount of nickel deposited on the filter was measured using atomic absorption spectrophotometry (Varian AA6). The nickel aerosol was produced with an ultrasonic nebuliser (DeVilbiss 35B). The mass median aerodynamic diameter, estimated by an impactor, was about 1 µm.<sup>15,17</sup>

### LUNG LAVAGE

Within three days after the last day of exposure the rabbits were killed by means of an overdose of sodium pentobarbital and the lungs were excised. Both lungs were lavaged with Hank's solution at about 37°C.<sup>18</sup> About 80 ml of the lavage fluid were collected.

### CELL SEPARATION

The separation procedure has been described previously.<sup>19</sup> Briefly,  $25-135 \times 10^6$  cells in 10 ml Hank's separation buffer were added to the elutriator system (Beckman JE-G elutriator rotor with a Beckman J21 B centrifuge) with an initial buffer flow rate of 32 ml/min. Material collected at this flow rate was referred to as fraction 1. Fractions 2 and 3 were collected at flow rates of 42 and 56 ml/min, respectively. The fraction volume was 100 ml. The rotor speed was

at  $2780 \pm 10$  rpm and the temperature 4°C. After stopping the rotor, fraction 4 was obtained by washing the separation chamber with 100 ml separation buffer at a flow rate of 80 ml/min.

### MORPHOLOGICAL MEASUREMENTS

The macrophages were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, postfixed in 1% OsO<sub>4</sub> in the same buffer, dehydrated in graded alcohol, and embedded in Polarbed 812. Thin sections were examined in a Jeol 100S electron microscope.

### LYSOZYME MEASUREMENTS

Lysozyme activity was studied using a modification described by Lundblad *et al*<sup>20</sup> of the agar heat killed *Micrococcus lysodeikticus* lysoplate technique.<sup>21</sup> The plates contained nine circular wells, 4 mm in diameter. A 10 µl sample was placed in each well. On each plate samples were placed in triplicate together with one standard sample of human lysozyme (the National Swedish Bacteriological Laboratory). In addition, lysozyme standards of 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, and 20.0 µg/ml were tested on separate plates. After 24 hours at 37°C the diameter of the clearance zone was measured.

### OXIDATIVE METABOLIC ACTIVITY

The oxidative metabolic activity of the macrophages was determined by measuring their ability to reduce nitroblue tetrazolium to formazan both at rest and in the presence of *Escherichia coli* bacteria.<sup>15</sup>

### STATISTICAL ANALYSIS

Lysozyme activity data were evaluated using Wilcoxon's U test or Wilcoxon's matched pairs signed ranks test and NBT test data were evaluated using a *t* test. Direction was not predicted in any of the tests.

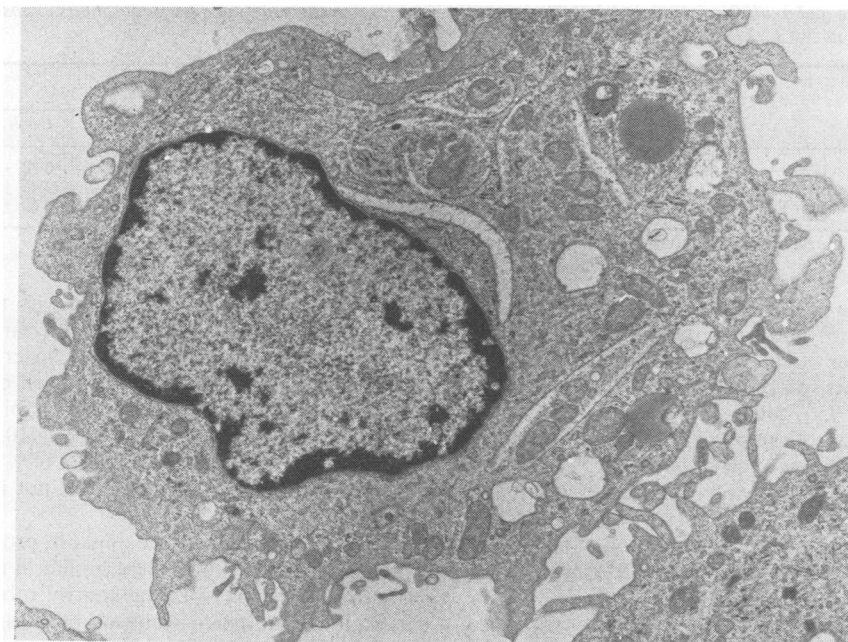
## Results

### GROSS FINDINGS

The lungs from the exposed rabbits and controls appeared to be essentially normal. Lung lavage fluid

Table 1 Percentage macrophages with 0-3, 4-10, and > 10 laminated inclusions from rabbits exposed to 0.6 mg/m<sup>3</sup> Ni<sup>2+</sup> as NiCl<sub>2</sub> for four to six weeks, five days a week, six hours a day and from controls. From the exposed rabbits unfractionated macrophages and fractionated macrophages were studied. (Data are given as mean  $\pm$  SD)

No of laminated inclusions/macrophage	Exposed macrophages				
	Controls (n = 7)	Unfractionated macrophages (%) (n = 8)	Macrophages from fraction 2 (%) (n = 5)	Macrophages from fraction 3 (%) (n = 5)	Macrophages from fraction 4 (%) (n = 7)
0-3	96 $\pm$ 2	59 $\pm$ 17	89 $\pm$ 5	78 $\pm$ 10	67 $\pm$ 17
4-10	3 $\pm$ 2	27 $\pm$ 8	12 $\pm$ 4	16 $\pm$ 6	26 $\pm$ 1
> 10	1 $\pm$ 1	14 $\pm$ 11	2 $\pm$ 1	7 $\pm$ 4	11 $\pm$ 8



**Fig 1** *Alveolar macrophage from fraction 2. No laminated inclusions are found in cytoplasm,  $\times 8000$ .*



**Fig 2** *Alveolar macrophage from fraction 3. In this cell four profiles of laminated inclusions are seen (arrow),  $\times 7000$ .*

Table 2 Lysozyme and oxidative metabolic (NBT test) activities in fractionated macrophages from rabbits exposed to 0.6 mg/m<sup>3</sup> of Ni<sup>2+</sup> as NiCl<sub>2</sub>. (Data are given as mean  $\pm$  SD).

	Lysozyme activity	Nitroblue tetrazolium reduction*	
	$\mu\text{g}/10^6$ macrophages	At rest	<i>E. coli</i> stimulation
Macrophages from fraction 2	0.9 $\pm$ 0.4 (n = 6)	0.13 $\pm$ 0.04 (n = 6)	0.32 $\pm$ 0.12 (n = 4)
Macrophages from fraction 3	1.0 $\pm$ 0.5 (n = 6)	0.20 $\pm$ 0.09 (n = 8)	0.45 $\pm$ 0.19 (n = 8)
Macrophages from fraction 4	1.7 $\pm$ 1.0 (n = 6)	0.27 $\pm$ 0.11 (n = 8)	0.60 $\pm$ 0.21 (n = 8)

\*Amount of formazan produced by  $3 \times 10^6$  macrophages during 30 minutes, expressed as optical density.

from many of the rabbits exposed to nickel and from one of the controls had a turbid or opaque appearance. The number of cells lavaged from the lungs of the control rabbits was  $37 \pm 15 \times 10^6$  and from the exposed rabbits,  $95 \pm 40 \times 10^6$  (mean  $\pm$  SD).

After elutriation of the cells from the exposed rabbits the largest number of cells were found in fraction 4, fewer cells in fraction 3, and fewer still in fraction 2. Fraction 1 contained surfactant structures and cell debris. As the number of cells in fractions 2 and 3 was often limited, all tests could not be performed for all rabbits.

#### MORPHOLOGY

Table 1 shows the distribution of laminated inclusions in the macrophages from the exposed rabbits

and the controls. The control rabbit with turbid lung lavage fluid had macrophages that clearly differed from the other controls and from the macrophages of the exposed rabbits. Dead cells and cell debris were present in the lavage fluid from this rabbit. The percentages of macrophages with 0–3, 4–10, and >10 laminated inclusions/cell profile were 68%, 18%, and 14% for this rabbit. These data are not included in table 1.

Fraction 2 contained the highest proportion of small macrophages which were similar in appearance to the macrophages from the control rabbits. These cells had 0–3 laminated inclusion profiles/cell profile and the cell surface had moderate protrusions (fig 1) (table 1). In fraction 3 there was a small increase in the proportion of macrophages with a moderate num-

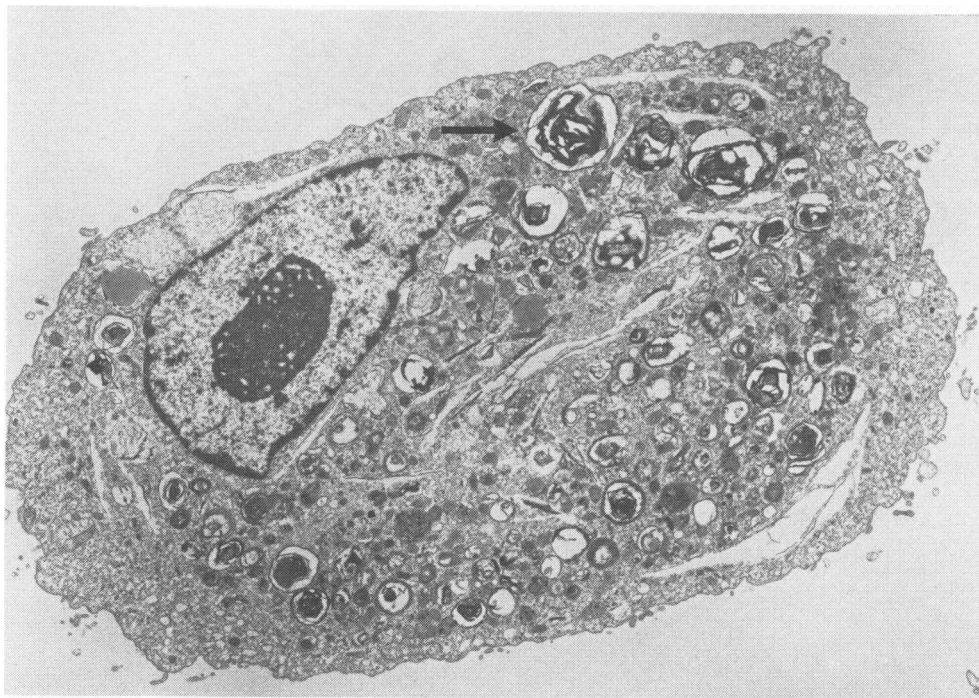


Fig 3 Alveolar macrophage from fraction 4. Many lamellated inclusions (arrow) are present in cytoplasm and surface lacks protrusions,  $\times 5000$ .

ber of laminated inclusions, 4–10 profiles/cell profile (fig 2), and in the proportion of macrophages with many inclusions, 10 profiles/cell profile (fig 3) (table 1). Fraction 4 contained the highest percentage of macrophages with laminated inclusions. Cells containing a moderate and high number of laminated inclusions were most frequent in this fraction. Cells with closely packed laminated inclusions and lipid droplets were rounded with a smooth surface (fig 3) (table 1).

#### LYSOZYME AND NBT ACTIVITIES

Lysozyme activity in the lung lavage fluid and in the macrophages was  $5.5 \pm 2.3$  (mean  $\pm$  SD) and  $6.4 \pm 2.5$  for the controls ( $n = 7$ ), respectively, and  $1.4 \pm 0.8$  and  $1.2 \pm 0.7$  for the exposed rabbits ( $n = 8$ ), respectively. Both activities were significantly lower in the nickel exposed animals than in the controls ( $p < 0.01$ ) (table 2). If the control with the morphologically changed macrophages is included the result is similar and the level of significance unchanged. For the fractionated macrophages from the exposed rabbits the lowest activity was found in fraction 2 and the highest in fraction 4. The activity in these two fractions differed significantly ( $p < 0.05$ ) (table 2).

In fractionated macrophages from the exposed rabbits the mean value of the NBT activity, both at rest and during *E coli* stimulation, increased gradually with the fraction number—that is, with the amount of intracellular surfactant (table 2). The activity in the macrophages from fraction 4 was significantly higher than that in the macrophages from fraction 2 both at rest and during *E coli* stimulation ( $p < 0.02$  and  $p < 0.05$ , respectively).

#### Discussion

The results show that the elutriation procedure is suitable for separating alveolar macrophages from nickel exposed rabbits with respect to size and content of surfactant inclusions. Macrophages from control rabbits are fewer in number, contain few surfactant inclusions, and are more equal in size.<sup>14 15</sup> In a pilot study where control macrophages were separated by elutriation most cells appeared in fractions 2 and 3 (unpublished data). Because of the small number of cells in the other fractions an investigation, comparable with that performed on macrophages from nickel exposed rabbits, was not feasible. Earlier studies have shown that inhalation exposure to nickel increases NBT activity in macrophages<sup>15 22</sup> and that surfactant material from nickel exposed rabbits increases the NBT activity in vitro.<sup>13</sup> In this study the NBT values correlated positively with the number of laminated structures in the fractionated cells from the nickel exposed rabbits. This finding supports the assumption

that the different amounts of laminated structures in the macrophages in the four fractions may be used to study causal relations between increases in surfactant material, induced by nickel exposure, and effects on alveolar macrophages in vivo.

The lysozyme activity in lavage fluid and macrophages was greatly decreased in the nickel exposed rabbits compared with the controls; this agrees with earlier results.<sup>78</sup> The decrease in lysozyme activity in the fractionated cells was greatest in fraction 2, whereas fraction 4, which contained the highest percentage of cells with many laminated inclusions, showed the lowest decrease in lysozyme activity. This indicates that the impairment of lysozyme activity is not related to increased amounts of surfactant material. Thus nickel probably exerts a direct effect on the alveolar macrophages resulting in an impaired lysozyme activity in the lungs.

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